

## REMARKS

Applicants respectfully request the Examiner to reconsider the rejections in view of the above amendment and the following remarks.

### I. Amendments to the Claims

Upon entry of the foregoing amendment, claims 1, 3, 6-14, 16-19, and 21-23 are pending in the Application. Claim 1 is amended.

Applicants respectfully request entry of the above amendment and submit that the above amendment does not constitute new matter. Claim 1 has been amended for the sole purpose of expediting prosecution by enhancing readability in the context of the subject matter. Support for the amendments to the claim can be found throughout the specification and in the claims as originally filed. In particular, support for the amendment to claim 1 can be found, *inter alia*, in the specification at ¶¶ [0049] and [0053].

Based on the instant amendment and remarks, Applicants respectfully request that the Examiner withdraw the outstanding rejections.

### II. Rejection under 35 U.S.C. § 112, ¶ 2

The Office Action stated that claims 1, 3, 6-14, 16-19, and 21-23 are rejected under 35 U.S.C. § 112, ¶ 2, as allegedly being indefinite. *See* Office Action, pp. 2-4. Applicants respectfully traverse this rejection.

The Office Action states, “Amended claim 1 (and dependent claims) remains vague and indefinite and as written is directed to action steps and elements which do not interrelate.” *See* p. 3. Applicants respectfully submit that the claim 1 is, in fact, clear and definite.

“Homologous polynucleotides” refer to a difference in at least one corresponding residue position with regard to two or more polynucleotides. *See* specification, ¶ [0019]. This may include single-stranded polynucleotides. On the other hand, “heteroduplex polynucleotides,” not to be confused with heterologous polynucleotides, refer to “double-stranded polynucleotides in which the two strands are not perfectly complementary to each other.” *See id.* at ¶ [0020]. “Homologous heteroduplex polynucleotides” refer to the situation where a residue position in a plurality of double-stranded polynucleotides differs from one another *and* the two strands of the

double-stranded polynucleotides are not perfectly complementary to each other. *See id.* at ¶ [0021]. An illustration showing examples of each is attached in Appendix A. However, to expedite prosecution, claim 1 has been amended to omit the term “homologous.”

The Office Action also states, “The homologous polynucleotides of 1(a) are parental polynucleotides which may also be homologous heteroduplex polynucleotide fragments even before the denaturing and hybridizing step of 1(b). In other words, the beginning substrate, i.e., the homologous polynucleotide, is a heteroduplex polynucleotide.” *See* p. 4.

Initially Applicants point out that claim 1 has been amended to replace “homologous” with “mutant” to avoid confusion. Second, the mutant polynucleotides are obtained *from* a parental polynucleotide by mutagenesis: they are not the parental polynucleotide as indicated in the Office Action. Finally, because “homologous” may refer to single *or* double-stranded polynucleotides, homologous polynucleotides may not be heteroduplex polynucleotides, which must be double-stranded.

For at least the reasons provided above, Applicants respectfully assert that claims 1, 3, 6-14, 16-19 and 21-23 are clearly definite and recite steps and elements that interrelate. Therefore, Applicants respectfully request the Examiner to withdraw the rejection of claims 1, 3, 6-14, 16-19 and 21-23 under § 112, ¶ 2.

### **III. Rejection under 35 U.S.C. § 102(b)**

The Office Action stated that claims 1, 3, 6, 7, 9, 12, 16, and 21 are rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Jyy-Jih Tsai-Wu *et al.*, *Preparation of Heteroduplex DNA Containing a Mismatch Base Pair with Magnetic Beads*, Analytical Biochemistry 275, 127-129 (1999) (“the Tsai-Wu reference”). *See* Office Action, pp. 4-7. Applicants respectfully traverse this rejection.

Applicants’ invention is directed to a method for obtaining polynucleotide fragments for use in polynucleotide shuffling. Applicants’ independent claim 1 encompasses the invention of: obtaining a library of mutant polynucleotides from a parental polynucleotide by mutagenesis, denaturing and hybridizing said mutant polynucleotides to form heteroduplex polynucleotides, cleaving said heteroduplex polynucleotides by using proteins of a polynucleotide repair system which cleave mismatched base pairs, and denaturing said cleaved heteroduplex polynucleotides to obtain said polynucleotide fragments.

Furthermore, paragraph [0053] of the specification states:

The initial library can also be produced by any method known to one skilled in the art, for example, by starting from a wild-type gene, by successive managed stages of mutagenesis, by error-prone PCR, by random chemical mutagenesis, by random mutagenesis *in vivo*, or by combining genes of close or relatively distant families within the same or different species. Preferably, the initial library results from chain polymerization reactions under conditions that create random, localized mutations.

Therefore, the goal of Applicant's invention is to produce a variety of fragments for use in polynucleotide shuffling.

By distinct contrast, the Tsai-Wu reference discloses a "method to produce DNA heteroduplex containing a mismatch base pair for the study of the mismatch repair gene products." Therefore, the goal is to prepare "the heteroduplex in large quantity with high purity." See p. 128, top of left column. Thus, the Tsai-Wu reference is *not* trying to produce mutations to provide a variety of fragments.

The Office Action stated the Tsai-Wu reference taught the use of M13mm3 and M13mm4 as a "template for PCR . . . and Taq polymerase is employed, a polymerase known to a person of skill in the art to introduce errors." See Office Action, p. 5. Applicants respectfully disagree.

First, the Tsai-Wu reference chose two M13mp18 phage derivatives, M13mm3 and M13mm4, because they have identical DNA sequences except for a single nucleotide, which allows the introduction of a G/A mismatch when mix together. M13mm3 provides the A strand whereas M13mm4 provides the complementary G strand. See the Tsai-Wu reference, p. 128, first full paragraph. The A and G strands were prepared by PCR using 5' and 3' primers with and without a biotin group. The unbiotinylated A and G strands were then recovered, renatured and annealed to form the G/A heteroduplex. See *id.* at p. 128, Fig. 1. Therefore, the G/A mismatch is purposely introduced, but not through the PCR process. PCR was used only to amplify the DNA sequences and *not* to introduce mutations.

Second, the Tsai-Wu reference uses KlenTaq DNA polymerase, *not* Taq polymerase. See the Tsai-Wu reference, p. 128, first full paragraph. KlenTaq polymerase is usually used in a mixture with a proofreading polymerase to provide greater fidelity in order to prevent mutations during the amplification. Product data sheets for KlenTaq polymerase from Clontech and Sigma-Aldrich, Inc. are attached in Appendix B.

Finally, even if errors were introduced in the DNA sequences in the method disclosed in the Tsai-Wu reference, the goal of the study was not to prepare a plurality of polynucleotide fragments, but to intentionally introduce a single G/A mismatch in a DNA sequence. In fact, the G/A mismatch-containing heteroduplex was purified and inspected for the presence of any homoduplex DNA. Two restriction sites were designed at the mismatch site so that T/A and G/C homoduplexes could be cleaved by the restriction enzymes NsiI and NheI, respectively. *See* the Tsai-Wu reference, p. 128, second full paragraph and p. 129, Fig. 2. MutY was used to specifically cleave the G/A mismatch. *See id.* The obtained products were then analyzed on a polyacrylamide gel and showed that “this preparation was indeed a G/A heteroduplex and did not contain either the T/A homoduplex or G/C homoduplex.” *See id.*

The Office Action also stated, “The cleavage products were then fractionated on a denaturing gel.” *See* p. 6. However, the aim of the polyacrylamide gel was to analyze the quality of the mismatch heteroduplex as discussed above, *not* to produce fragments for use in polynucleotide shuffling. Moreover, the Tsai-Wu reference does not recover the fragments from the gel.

For at least these reasons, the Tsai-Wu reference does not anticipate the claims of the present application. Therefore, Applicants respectfully request that the rejection of claims 1, 3, 6, 7, 9, 12, 16, and 21 under 35 U.S.C. § 102(b) be reconsidered and withdrawn.

### CONCLUSION

All of the stated grounds of rejection have been properly traversed. Applicants, therefore, respectfully request that the Examiner reconsider all presently outstanding rejections and that the rejections be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance.

The Examiner is respectfully requested to contact the undersigned by telephone at the below listed telephone number, in order to expedite resolution of any issues and to expedite passage of the present application to issue, if any comments, questions, or suggestions arise in connection with the present application.

Respectfully submitted,

Dated: Aug. 16, 2006

By:

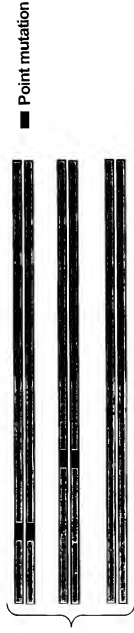
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# **APPENDIX A**

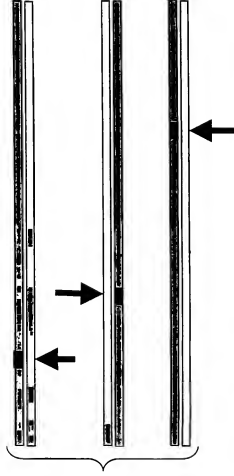
Homologous  
polynucleotides



Heteroduplex  
polynucleotides



Homologous  
Heteroduplex  
polynucleotides



Confidential

proteus

# **APPENDIX B**



# Clontech

United States

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## Product Catalog

Cart Summary  
You have 0 Product(s) in your Shopping  
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Search: 

### KlenTaq LA Polymerase Mix

KlenTaq LA Polymerase Mix is designed for long and accurate amplification of PCR products. It contains a primary polymerase, KlenTaq-1, and a minor amount of proofreading polymerase.

#### Applications

- Long and accurate PCR
- cDNA PCR
- Preparative PCR

#### Notice To Purchaser:

PCR

1					
Name	Size	Cat. No.	Price	MF Price	Shop
KlenTaq LA Polymerase Mix	100 rxns	639108	N/A	N/A	

**www.sigma-aldrich.com**

Product Name or No.

## D5062 KlenTaq® LA DNA Polymerase Mix

Sigma



[Expand/Collapse All](#)

### Price and Availability

Product Number	Your Price EUR	Available to Ship	Quantity	Actions
D5062-1.5KU	718,00	19.07.2006 details...	<input type="text"/>	
D5062-125UN	82,80	19.07.2006 details...	<input type="text"/>	
D5062-500UN	281,00	19.07.2006 details...	<input type="text"/>	

### Descriptions

Application	KlenTaq® LA DNA Polymerase provides an excellent alternative to Taq DNA Polymerase for intermediate length products. It allows higher yields and greater fidelity (up to 4× that of Taq). It can amplify genomic DNA up to 5 kb or less complex DNA targets such as bacterial, viral targets or cDNA up to 20 kb. With its increased thermostability, it is the ideal choice for amplifying GC-rich regions or templates with difficult secondary structure.
Features and Benefits	<ul style="list-style-type: none"> <li>• KlenTaq LA DNA Polymerase has increased thermostability and processivity, resulting in increased yields</li> <li>• Amplify difficult structure or GC-rich templates. The increased thermostability allows higher temperature conditions to disrupt difficult secondary structures</li> <li>• Increased fidelity at up to 4× higher than that of Taq DNA polymerase</li> <li>• Tolerance to a broad range of magnesium concentrations eliminates the need to optimize <math>MgCl_2</math></li> <li>• Amplify up to 5 kb genomic targets and up to 20 kb on less complex targets, such as lambda DNA</li> </ul>
Other Notes	KlenTaq LA DNA Polymerase Mix is an optimized mixture combining KlenTaq-1 with a <u>proofreading enzyme</u> . KlenTaq-1 is a Klenow-fragment analog of Taq DNA Polymerase. It has no endonuclease or exonuclease activity, but is more thermostable than Taq or other terminal deletions of Taq. Since a wide range of magnesium concentration is tolerated by this enzyme, generally no magnesium optimization is needed. The proofreading polymerase provides the 3'→5' exonuclease activity that is necessary for longer and higher fidelity products.
Packaging	KlenTaq LA DNA polymerase is provided with an optimized 10× reaction buffer.
Unit Definition	One unit will incorporate 10 nmol of total dNTPs into acid precipitable DNA in 30 min. at 74 °C.
Legal Information	<p>KlenTaq is a registered trademark of Wayne Barnes.</p> <p>Purchase of this product is accompanied by a limited license for use in the Polymerase Chain Reaction (PCR) process for research purposes only and in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by an up-front license fee, either by payment to Applied Biosystems or as purchased, i.e., and authorized thermal cycler. KlenTaq is a registered trademark of Wayne Barnes. Licensed under U.S. Patent Number 5,436,149 owned by Takara Shuzo Co., Ltd.</p>
Properties	
concentration	5 units/μL
shipped in	wet ice
storage temp.	-20°C
References	
Reference	Barnes, W.M., PCR amplification of up to 35-kb DNA with high fidelity and high yield from bacteriophage templates. <i>Proc. Natl. Acad. Sci. USA</i> 91, 2216-2220, (1994)
Safety	
Hazard Codes	Xi
Risk Statements	36/37/38
Safety Statements	26-36

**Related Categories**

... [PCR/Amplification](#) > [Long & Accurate PCR](#)

... [Nucleic Acid Amplification](#) > [Long and Accurate PCR](#)

## Product Information

**KLENTAQ LA CORE KIT**  
 Product Number KLENCOR  
 Technical Bulletin No. MB-620  
 Storage Temperature -20°C  
 March, 2000

### TECHNICAL BULLETIN

#### Introduction

The Polymerase Chain Reaction (PCR)<sup>1</sup> is a powerful molecular biology technique now in standard use for cloning, sequencing and genome mapping. The primary enzyme used in PCR is Taq DNA polymerase. However, Taq DNA polymerase is generally limited to amplifications up to 5 kb in part because Taq DNA polymerase has no 3'→5' exonuclease or "proofreading" activity, which means periodic misincorporations are not repaired. After a misincorporation has taken place, the enzyme will either continue to incorporate nucleotides, causing a processive mistake, or a terminal event will occur and elongation will be arrested. Long and Accurate (LA) PCR combines a highly processive thermostable polymerase with a second thermostable polymerase that exhibits a 3'→5' exonucleolytic activity. This blend increases the length of amplification products by using the proofreading polymerase to repair terminal misincorporations. This repair allows the polymerase to resume elongating the growing DNA strand.

KlenTaq LA is a specially blended enzyme mix containing KlenTaq-1 DNA polymerase (a 5'-exo-minus, N-terminal deletion of Taq DNA polymerase) and a small amount of a proofreading DNA polymerase. This blending of KlenTaq-1 and a proofreading polymerase increases the fidelity, yield and the length of the amplified product. KlenTaq-1 is more efficient and more processive than either native Taq DNA polymerase or other N-terminal deletions of Taq. This means the same degree of amplifications can be achieved with fewer cycles. KlenTaq LA has a broad magnesium optimum, so it is typically unnecessary to optimize the magnesium concentration in the reaction mixtures. It has fidelity four times greater than that seen in standard Taq DNA polymerase. KlenTaq LA is ideal for DNA amplifications 0.5-5 kb in length on genomic DNA and up to 10 kb on less complex templates.

Sigma's KlenTaq LA Core Kit includes all the reagents necessary to perform long PCR on genomic, bacterial, viral or cloned DNA templates with KlenTaq LA Polymerase Mix.

#### Reagents Provided

(Reagents provided are sufficient for 25 reactions.)

- KlenTaq LA Polymerase Mix 125 units  
 Product No. D5187, 5 units/μl in 50% glycerol, 40 mM Tris-HCl (pH 7.5), 50 mM KCl, 25 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1 mM EDTA, 5.0 mM 2-mercaptoethanol, 0.25% Thesit (polyoxyethylene 9 lauryl ether). A proofreading DNA polymerase is a minor component of KlenTaq LA polymerase mix.
- 10X Buffer for KlenTaq LA 1.5 ml  
 Product No. B6178, 400 mM Tricine-KOH (pH 9.2 at 25°C), 150 mM KOAc, 35 mM Mg(OAc)<sub>2</sub>, 750 μg/ml Bovine Serum Albumin
- Deoxynucleotide Mix, Product No. D7295 0.2 ml  
 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP
- PCR Water, Product No. W1754 1.5 ml
- Control Template, 25 μl  
 Product No. D8920, lambda DNA, 2.5 μg/μl
- Control Primer B, (Forward), 25 μl  
 Product No. P7604, 10 pmol/μl  
 5' CAC AAC GGA ACA ACT CTC ATT 3'
- Control Primer A, (Reverse), 25 μl  
 Product No. P7729, 10 pmol/μl  
 5' CAG CAA TAC AGG GAA AAT CTT 3'

**Materials and Reagents Required but Not Provided**  
 (Sigma Product Numbers have been given where appropriate.)

- Specific Primers
- DNA to be amplified
- Mineral Oil, Product No. M8662
- Dedicated pipettes
- PCR pipette tips
- 0.2 or 0.5 ml thin-walled PCR microcentrifuge tubes
- Thermal cycler

#### Precautions and Disclaimer

Sigma's KlenTaq LA DNA Polymerase is for laboratory use only; not for drug, household or other uses.

#### Storage

Store at -20°C. Storage in "frost-free" freezers is not recommended.

### Reaction Optimization

The optimal conditions for amplification will vary based on the template DNA, primers, experimental protocols, tubes and thermal cyclers. Reliable amplification of long DNA sequences requires: 1) effective denaturation of DNA template, 2) adequate extension times to produce large products and 3) protection of target DNA from damage by depurination. Depurination during cycling is minimized by the use of a buffer with a pH greater than 9.0 at 25°C. Effective denaturation is accomplished by the use of higher temperatures for shorter periods of time or by the use of co-solvents, such as dimethyl sulfoxide. Addition of DMSO in the reaction at a final concentration between 1 to 4% may increase yield and improve reliability of the system with some complex PCR targets. Betaine (0.8-1.3 M) has been reported to improve the amplification of DNA by reducing secondary structure in GC-rich regions (Rees et al., 1993).

### Thermal Cycler

Perkin-Elmer DNA Cyclers 480, 2400 and 9600 have been used to develop cycling parameters. Other types of thermal cyclers can also be used, but may require further optimization of cycling parameters.

### Primer design

Primer design is critical for successful long PCR. Primers are usually 21 to 34 bases in length and designed to have a GC content of 45-50%. In general, primers should have a  $T_m$  of at least 70°C if a 68°C annealing/extension step is used. Optimally, the melting temperatures of the forward and reverse primers should be within 3°C of each other and the  $T_m$  of the primers should be between 65° and 72°C. Primers should not have any internal base-pairing sequences (i.e., potential hairpins) or any significant length of complementary regions between the two PCR primers. It may be helpful to design primers with a final CC, GG, CG, or GC on the 3' end of the primers in order to increase priming efficiency (Lowe, 1990).

### Template

KlenTaq LA can tolerate a wide range of template quality. However, as the length of the target increases, the quality of the target becomes more important. High quality and adequate length of the template are essential for reliable amplification of larger fragments. The number of full-length unnickeds decreases as the length of the target increases. Nicked or damaged DNA can serve as a potential priming site resulting in high background. Extreme care must be taken in the preparation and handling of the DNA target for long PCR. Avoid freezing or alternatively, freeze only once to minimize damage.

### Magnesium concentration

Optimization of magnesium concentration is not necessary with KlenTaq LA. KlenTaq has been found to be insensitive to magnesium concentration (Barnes, 1994).

### Cycle Conditions

Extension temperature should be limited to 68°C for optimal performance. Temperatures greater than 68°C may result in a reduced amount or no product. Primer annealing and product extension can also be combined into one step if primers are designed to have a  $T_m$  between 65° and 72°C. Cycles for denaturation parameters should be kept short to minimize both damage to the template and inactivation of the enzyme. The half life of the enzyme is markedly decreased at temperatures above 96°C.

### Touchdown PCR

"Touchdown" PCR has been shown to significantly improve the specificity of many PCR reactions in a wide variety of applications (Don et al, 1991). Briefly, touchdown PCR uses an annealing/extension temperature that is several degrees (typically 3-10°C) higher than the  $T_m$  of the primers during the initial cycles (typically 5-10). The annealing/extension temperature is then reduced to the primer  $T_m$  for the remaining cycles.

### Amplification Procedure

1. Add the following reagents to a thin-walled 0.2 or 0.5 ml microcentrifuge tube:

Volume	Reagent	Final Concentration
5.0 µl	10X PCR Buffer for KlenTaq LA	1X
1.0 µl	dNTP Mix (10 mM each dNTP)	200 µM (each dNTP)
3.0 µl	Forward Primer (10 pmole/µl)	600 nM
3.0 µl	Reverse Primer (10 pmole/µl)	600 nM
3.0 µl	Control Template DNA (2.5 ng/µl)	0.15 ng/µl (~300 pM)
34.0 µl	Water	-
1µl	KlenTaq LA Polymerase	0.1 unit/µl
50 µl Total Volume		

Note: Less DNA template is required if amplifying plasmid DNA (typically 1-5 ng). If amplifying from a genomic "pool", more DNA template may be necessary.

- Mix gently by vortex and briefly centrifuge to collect all components to the bottom of the tube.
- Add 50  $\mu$ l of mineral oil to the top of each tube to prevent evaporation (optional, depending on model of thermal cycler).
- The amplification parameters will vary depending on the primers and the thermal cycler used. It may be necessary to optimize the system for individual primers, template, and thermal cycler.

Common cycling parameters based on length of target

	<5 kb	5-10 kb
Initial denaturation	95°C 1 min	95°C 1 min
For Cycles 1 to 25/35		
Denaturation	94°C 30 sec	94°C 30 sec
Annealing/Extension	68°C 3-5 min	68°C 6-10 min
Final extension (optional)	68°C 3 min (may reduce background)	68°C 10 min
Hold	4°C	4°C

- The amplified DNA can be evaluated by 0.8 – 1.0% agarose gel electrophoresis and subsequent ethidium bromide staining (Sambrook, 1989). The expected PCR product is a 10 kb fragment, corresponding to position 37272-47187 of Lambda genomic DNA (Genbank accession number J02459).

Notes:

- For most applications, two step cycles are recommended over three step cycles. A two step cycle involves denaturation at  $T_1$ , followed by annealing and extension at  $T_2$ . A three-step cycle has separate temperatures for denaturation, annealing and extension. Three step PCR is more flexible and is necessary when the  $T_m$  of the primers is less than 70°C.

Below is a sample three step PCR based on amplification of a 10 kb lambda fragment.

Three Step PCR for 10 kb targets		
Initial Denaturation	95°C	1 min
For Cycles 1 to 25/35		
Denaturation	95°C	30 sec
Annealing	55°C	15 sec
Extension	68°C	10 min
Final extension (optional)	68°C	10 min
Hold	4°C	

- The number of cycles will depend on the abundance of target DNA. For multiple copy genes or medium to high abundance cDNAs, 25 cycles is sufficient. For single or low copy number genes or rare cDNAs, 30-35 cycles is recommended.
- Denaturation time should be as short as possible. In some cases, better results are seen with a 15 second denaturation time at 95°C. Exposure of DNA to high temperatures causes some depurination of single-stranded DNA during denaturation, which eventually leads to strand scission. Although KlenTaq-1 is extremely stable at elevated temperatures of 95°C, high temperatures can lead to a gradual loss of enzymatic activity. Minimizing denaturation time is particularly important in experiments with very long templates where total cycling time can exceed 12 hours.

† The PCR process is covered by patents owned by Hoffman-LaRoche, Inc.

## References

- Barnes, W.M. PCR amplification of up to 35-kb DNA with high fidelity and high yield from bacteriophage templates. *Proc. Natl. Acad. Sci. USA* 91:2216-2220 (1994)
- Cheng, S., *et al.* Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci. USA* 91:5695-5699 (1994)
- Don, R.H. *et al.* 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* 19:4008 (1991)
- Lowe, T. *et al.* A computer program for selection of oligonucleotide primers for polymerase chain reaction. *Nucl. Acids Res.* 18(7): 1757-1761 (1990)
- Rees, W.A., *et al.*, Betaine can eliminate the base pair composition dependence of DNA melting. *Biochemistry*, 32, 137-144 (1993)
- Roux, K.H. Optimization and troubleshooting in PCR. *PCR Methods Appl.* 4:5185-5194 (1995)
- Rychlik, W., and Rhoads, R.E. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA, *Nucl. Acids Res.* 17:8543-8551 (1989)
- Sambrook, J, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, New York (1989) (Product No. M3401)

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## Troubleshooting Guide

Symptoms	Possible Causes	Comments
<b>No PCR product is observed</b>	a) A PCR component may be missing or degraded.	A positive control should always be run to insure components are functioning. A checklist is also recommended when assembling reactions.
	b) There may be too few cycles performed.	Increase the number of cycles (3-5 additional cycles at a time).
	c) The annealing temperature may be too high.	Decrease the annealing temperature in 2-4°C increments.
	d) The primers may not be designed optimally.	Confirm the accuracy of the sequence information. If the primers are less than 22 nucleotides long, try to lengthen the primer to 25-30 nucleotides. If the primer has a GC content of less than 45%, try to redesign the primer with a GC content of 45-60%.
	e) There may not be enough template.	After increasing the number of cycles has shown no success, repeat the reaction with a higher concentration of template.
	f) The template may be of poor quality.	Evaluate the template integrity by agarose gel electrophoresis. It may be necessary to repurify template using methods that minimize shearing and nicking.
	g) The denaturation temperature may be too high or too low.	Optimize the denaturation temperature by increasing or decreasing the temperature in 1°C increments.
	h) The denaturation time may be too long or too short.	Optimize the denaturation time by increasing or decreasing it in 10 second increments.
	i) The extension time may be too short.	Increase the extension time in 1 minute increments, especially for long templates.
	j) The reaction may not have enough enzyme.	One $\mu$ l (5 units) is sufficient for most applications. It is recommended that the cycling parameters be optimized before the enzyme concentration is increased. In rare cases, the yields can be improved by increasing the enzyme concentration. However, if the enzyme concentration is above 2 $\mu$ l (10 units), higher background levels may be seen.
	k) Magnesium levels may be too low.	This is unlikely if the 10 X-reaction buffer (provided) is used and the deoxynucleotides do not exceed a concentration of 0.2 mM each. This is due to KlenTaq-1's broad magnesium optimum. However, if the concentration of EDTA in the sample is greater than 5 mM, this can reduce the effective concentration of magnesium.
	l) Deoxynucleotides are too low.	This is unlikely if the final concentration of each deoxynucleotide is 0.2 mM. This concentration of dNTPs is suitable for a wide range of applications. If the dNTPs are being prepared in the laboratory, be sure that the final concentration of each deoxynucleotide is 0.2 mM. If the concentration of dNTPs is increased, the magnesium concentration will need to be increased proportionately.
	m) Target template is difficult.	In most cases, inherently difficult targets are due to unusually high GC content and /or secondary structure. 2-Betaine has been reported to help amplification of high GC content templates at a concentration of 0.8-1.3 M. In some cases, the addition of 2-5% DMSO may help.



Multiple Products	a) There may be too many cycles performed.	By reducing the cycle number, the nonspecific bands may be eliminated.
	b) The annealing temperature may be too low.	Increase the annealing/extension temperature in increments of 2-3°C.
	c) The primers may not be designed optimally.	Confirm the accuracy of the sequence information. If the primers are less than 22 nucleotides long, try to lengthen the primers to 25-30 nucleotides. If the primer has a GC content of less than 45%, try to redesign the primers with a GC content of 45-60%.
	d) Touchdown PCR may be needed.	"Touchdown" PCR significantly improves the specificity of many PCR reactions in various applications. Touchdown PCR involves using an annealing/extension temperature that is higher than the $T_m$ of the primers during the initial PCR cycles. The annealing/extension temperature is then reduced to the primer $T_m$ for the remaining PCR cycles. The change can be performed in a single step or in increments over several cycles.
Products are smeared	a) Too many cycles may have been performed.	Reduce the cycle number in 3-5 cycle increments.
	b) The denaturation temperature may be too low.	Increase the denaturation temperature in 1°C increments.
	c) The extension time may be too long.	Decrease the extension time in 1-2 minute increments.
	d) Touchdown PCR may be needed.	See d. under "Multiple Products" for procedure.
	e) There may be too much enzyme in the reaction mix.	One $\mu$ l (5 units) is sufficient for most applications. However, this concentration may be too high for some applications. It is recommended the cycling parameters be optimized first, as described above, then reduce the enzyme concentration to 0.5-0.2X.
	f) Magnesium concentration may be too high.	KlenTaq-1 has a broad magnesium optimum and the magnesium concentration has been optimized in the supplied in the 10X-reaction buffer. If the 10X-reaction buffer is being used and the final concentration of each of the dNTPs is 0.2 mM, it is very unlikely the magnesium concentration is too high.
	g) The template concentration may be too high.	Reduce the concentration of the template in the PCR reaction.
Contamination	Contamination usually results in extra bands or smearing. It is recommended to include a water control (in place of the DNA template) in every PCR reaction to determine if the reagents used in the PCR reaction are contaminated with a template from a previous reaction.	
	When performing PCR directly on phage plaques or bacterial colonies, failure to isolate single plaques or colonies can produce multiple bands.	

#### Related Products

- PCR Optimization Kit, Product No. OPT-2
- Lambda HindIII DNA Marker, Product No. D9780
- BlueView™ Nucleic Acid Stain, Product Nos. T8935 and T9060, when added to agarose gel and running buffer, BlueView instantly stains nucleic acids
- Enhanced Avian RT-PCR kits, Product Nos. RTPCR-20 (20 reactions), RTPCR-100 (100 reactions), combines two powerful techniques to convert mRNA into cDNA and subsequently to amplify the cDNA and offers an enhanced ability to transcribe through difficult secondary structure at elevated temperatures (up to 65°C)

#### PCR Related Tware

- PCR Multiwell Plates, 96-well, Product No. Z37,490-3
- PCR Multiwell Plates, 384-well, Product No. Z37,491-1
- PCR Microtubes, 0.2 ml with attached caps, Product No. Z37,487-3
- PCR Microtubes, 0.2 ml strip tubes with strip caps, Product No. Z37,496-2
- Sealing accessory for PCR vessels, Micro Mats, Product No. Z37,493-8, molded to fit standard 96-well plates
- Sealing accessory for PCR vessels, Pierceable cap strips, Product No. Z37,495-4, caps in strips of eight, the center of each can be pierced with a hypodermic needle for quick sample removal without generating aerosols or other sources of cross-contamination. Caps can be used with 0.2 ml PCR strip and 96-well plates. Pkg of 120 strips (960 caps).
- PCR Workstation, 120V, Product No. Z37,621-3
- PCR Workstation, 240V, Product No. Z37,622-1

#### PCR Books

- PCR, 2<sup>nd</sup> ed, C.R. Newton and A. Graham, Bios Scientific Publishers, Oxford, England, 1997, Product No. Z37,831-3
- PCR: Essential Data Series. C.R. Newton, Ed., John Wiley and Sons, Inc., New York, NY, 1995, Product No. Z36,491-6

- PCR: A Practical Approach, M.J. McPherson, P. Quirke and G.R. Taylor, Eds., IRL Press, Oxford, England, 1991, Product No. P7186
- PCR 2: A Practical Approach, M.J. McPherson and B.D. Hames, Eds., IRL Press at Oxford University Press, Inc., Oxford, England, 1995, Product No. Z36,238-7
- PCR 3: PCR In Situ Hybridization, C.S. Herrington and J.J. O'Leary, Eds., IRL Press at Oxford University Press, Inc., Oxford, England, 1997, Product No. Z37,839-9
- PCR Cloning Protocols: Methods in Molecular Biology, Vol. 67, B. White, Ed., Humana Press, Totowa, NJ, 1996, Product No. Z37,422-9
- PCR In Bioanalysis: Methods in Molecular Biology, Vol. 92, S.J. Smeltzer, Ed., Humana Press, Totowa, NJ, 1998, Product No. Z37,960-3
- PCR Primer: A Laboratory Manual, C. Dieffenbach and G.S. Dvekster, Eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1995, Product No. Z36,411-8
- PCR Protocols: Current Methods and Applications: Methods in Molecular Biology, Vol. 15, B.A. White, Ed., The Humana Press, Totowa, NJ, 1993, Product No. Z35,736-7
- PCR Protocols: A Guide to Methods and Applications, M.A. Innis, et al., Eds., Academic Press, San Diego, CA, 1990, Product No. P8177
- PCR Protocols for Emerging Infectious Diseases, D.H. Pershing, Ed., American Society for Microbiology, Washington, DC, 1996, Product No. Z36,991-8
- PCR Sequencing Protocols, R. Rapley, Humana Press, Totowa, NJ, 1996, Product No. Z37,381-8
- PCR Strategies, Michael A. Innis, David H. Gelfand, and John J. Sninsky, Eds., Academic Press, San Diego, CA, 1995, Product No. Z36,445-2
- PCR Technology, Current Innovations, H.G. Griffin and A.M. Griffin, Ed., CRC Press, Boca Raton, FL, 1994, Product No. Z35,749-9
- PCR Technology: Principles and Applications for DNA Amplification, H.A. Erlich, Ed., IRL Press at Oxford University Press, Oxford, England, 1989, Product No. P3551

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